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Estradiol suppresses phosphorylation of ERα serine 167 through upregulation of PP2A in breast cancer cells

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Abstract. Aromatase inhibitors (AIs) are effective endocrine therapeutics for postmenopausal women with estrogen receptor (ER)α-positive breast cancer. However, the efficacy of the treatment is often limited by the onset of AI resistance, owing to the phosphorylation of ERα serine 167 (Ser167). Previous studies have indicated that hyperactivation of the phosphoinositide-3 kinase/RAC serine/threonine-protein kinase signaling pathway occurs in AI-resistant breast cancer models, which coincides with elevated levels of ERα phosphorylation at Ser167. The tumor suppressor serine/threonine-protein phosphatase 2A (PP2A) regulates the phosphatidylinositol 3-kinase/RAC serine/threonine-protein kinase signaling pathway. A previous study indicated that PP2A inhibition decreased ERα Ser167 phosphorylation and estradiol (E_2)-independent cell growth. The present study investigated the potential relevance of PP2A in E_2 deprivation-resistant MCF-7 cells. E_2 depletion reduced the susceptibility of MCF-7 cells to inhibitors of mechanistic target of rapamycin (mTOR) and significantly increased ERα Ser167 phosphorylation and decreased expression of PP2A. Conversely, long-term E_2-deprived (LTED) MCF-7 cells, a model of AI-resistant breast cancer, exhibited decreased ERα Ser167 phosphorylation and further upregulation of PP2A in E_2-containing medium. The PP2A activator forskolin (FSK) significantly inhibited LTED cell proliferation by increasing the effect of everolimus (Eve), an mTOR inhibitor. In summary, the present study provides further evidence that PP2A represents a therapeutic target for AI-resistant breast cancer.

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Key words: breast cancer, aromatase inhibitor, estrogen receptor, protein phosphatase 2A

Introduction

Estradiol (E_2) has an essential role in the development and progression of estrogen receptor (ER)-positive breast cancer (1,2). Therefore, the use of aromatase inhibitors (AIs), including letrozole, anastrozole and exemestane, as adjuvants is regarded as a standard approach in postmenopausal women with ER-positive breast cancer (3-5). However, certain patients with breast cancer develop resistance to AIs following long-term treatment (6). Previous studies have revealed cross-talk between the activation of the insulin-like growth factor-1 (IGF-I) signaling pathway and ERα in long-term AI-treated breast cancer cells (7,8). One mechanism of AI resistance is aberrant signaling through the phosphatidylinositol 3-kinase (PI3K)/RAC serine/threonine-protein kinase (Akt)/mechanistic target of rapamycin (mTOR) signaling pathway (8,9) (Fig. 1A).

Accordingly, the interruption of PI3K/Akt/mTOR signaling has been demonstrated in preclinical E_2-deprivation resistance models, in which an mTOR inhibitor in combination with exemestane led to abrogation of proliferation, induction of apoptosis and enhanced tumor regression (10). A substrate of mTOR complex 1, S6 kinase 1 (S6K), phosphorylates activation function domain 1 of ERα, which is responsible for ligand-independent receptor activation (7,8,11). IGF-1-dependent activation of ERα was proposed as the reason for AI resistance, and the role of S6 K was elucidated in previous studies (7,12). Abnormal activation of ERα is dependent on the phosphorylation of Ser104, Ser106, Ser118 and Ser167, located in the amino terminal A/B domain of ERα (13,14). The phosphorylation level of proteins is determined by the activity and balance of protein kinases, and phosphatases. Using the phosphatase inhibitor okadaic acid (OA) (15,16), a previous study demonstrated that serine/threonine-protein phosphatase 2A (PP2A) has an important role in the regulation of ERα Ser167 phosphorylation and in the proliferation of MCF-7 cells (17).

PP2A is a key tumor suppressor that regulates signaling pathways relevant to a number of types of human cancer (18,19). PP2A is a ubiquitously expressed member of a phosphoserine- and phosphothreonine-specific protein phosphatase family involved in the regulation of cell proliferation, cell differentiation, RNA transcription, DNA repair and apoptosis (20-22). As inhibition of its activity and loss of certain functional subunits are characteristics of neoplastic transformation, PP2A is widely

designated as a tumor suppressor (23). Forskolin (FSK) lacks adenylate cyclase-activating function but retains the ability to activate PP2A, which is necessary for growth inhibition and induction of apoptosis induction in leukemic cells (23).

In the present study, E_2 depletion decreased PP2A expression and reduced the susceptibility of MCF-7 cells to mTOR inhibitors. Furthermore, activation of PP2A by FSK enhanced the effect of everolimus (Eve) and strongly inhibited long-term E_2-deprived (LTED) cell proliferation.

Materials and methods

Cell culture. Human ER-positive breast cancer MCF-7 cells (American Type Culture Collection., Manassas, VA, USA) were maintained in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Inc., Tokyo, Japan) and 1% penicillin/streptomycin at 37˚C in a 5% CO_2-humidified atmosphere incubator. Cells treated with 17β-estradiol (E_2) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), Phos STOP (Sigma-Aldrich; Merck KGaA), OA, calyculin A (CalA), rapamycin and Eve (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in Dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd.) were cultured in phenol-red-free RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% dextran-coated charcoal (DCC)-treated FBS (Nichirei Biosciences, Inc.) and 1% penicillin/streptomycin. MCF-7 cells cultured in phenol-red-free RPMI 1640 with 10% dextran-coated charcoal (DCC)-treated FBS and 10 nM E_2 and then for 5 days without E_2 (MCF-7 5d) and 6 months without E_2 (LTED) were used in the experiment. LTED cells modeling AIs resistance were derived from a parental cell line by long-term culture in the presence of RPMI 1640 medium containing 10% DCC-treated FBS, as described previously (12,24,25). MCF-7 cells were cultured with E2 (10 nM), OA (100 nM), Cal A (1 nM), FK506 (10 nM), or DMSO (0.1%, vehicle) in phenol red-free RPMI 1640 medium supplemented with 10% dextran-coated charcoal fetal bovine serum for 5 days at 37˚C. The cell viability of cultured cells was determined using Cell Counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol.

Western blot analysis. Whole-cell lysates were collected using lysis buffer [containing 62.5 mM Tris HCl pH 6.8, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate, 5% sucrose and 0.01% Bromophenol Blue (Wako Pure Chemical Industries, Ltd.)]. The protein content was subsequently determined using a RC DC™ Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin (Sigma-Aldrich; Merck KGaA) as the standard. For western blot analysis, solubilized proteins (5 μg of protein/lane) were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare, Chicago, IL, USA). Membranes were pre-incubated with ImmunoBlock (DS Pharma Biomedical Co., Ltd. Osaka, Japan) as a blocking regent at room temperature for 30 min and then incubated at 4˚C overnight with antibodies at 1:1,000 dilution directed against Akt (cat. no. 9272S) and phosphorylated Akt Ser473 (cat. no. 4060S); Cell Signaling Technology, Inc., Danvers, MA, USA, ERα (cat. no. sc-543), phosphorylated ERα Ser167 (cat. no. sc-101676), and ERα Ser118 (cat. no. sc-101675), or a b-actin antibody (cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA). The membrane was subsequently washed with TBS-Tween 20 (TBS-T) buffer (20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 0.5% Tween-20) and incubated with a horseradish peroxidase-labeled secondary anti-rabbit (cat. no. 170-6515; Bio-Rad Laboratories, Inc., Hercules, CA, USA) or anti-mouse (cat. no. 330; MBL, Nagoya, Japan) IgG antibody for 1 h at room temperature. All antibodies were diluted in Can Get Signal Immunoreaction Enhancer solution (cat. no. NKB-101; Toyobo Life Science, Osaka, Japan). Once the membrane was washed with TBS-T buffer, immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP substrate (EMD Millipore, Billerica, MA, USA). The intensity of the chemiluminescence of specific bands was digitized using Cool Saver software version 1.2 (ATTO Corporation, Tokyo, Japan) and quantified.

Statistical analysis. All experimental data comparing more than two groups were analyzed by one-way analysis of variance followed by Fisher's protected least significant difference test. The software used for statistical analyses was SPSS v24 (IBM SPSS, Armonk, NY, USA). When differences were significant, subsequent analyses with post hoc t-tests with Bonferroni correction were performed. Other statistical comparisons were conducted by a two-tailed unpaired t-test. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

17β-estradiol depletion reduces the sensitivity to mTOR inhibitor treatment. MCF-7 cells have previously been used as a model for the study of the E_2 response *in vitro* (26,27). *In vitro* studies using E_2 deprivation or chronic exposure to anti-E_2 have led to the isolation of hormone therapy-resistant variants of MCF-7 cells (12,24,25). LTED cells serve as a model of AIs-resistant breast cancer, and have been generated by several laboratories (25). When MCF-7 cells were cultured in a phenol-red-free RPMI 1640 with 10% dextran-coated charcoal (DCC)-treated FBS medium, ERα Ser167 phosphorylation decreased in a time-dependent manner (Fig. 1B). Next, MCF-7, MCF-7 5d and LTED cells were evaluated for sensitivity to mTOR inhibition. MCF-7, MCF7 5d and LTED were treated with various amounts of the mTOR inhibitor rapamycin (concentrations of 1, 10, 100 or 1,000 nM) for 1 h at 37˚C, and the number of cells was measured with a Cell Counting kit 8. Following treatment of the cells with 1 nM rapamycin for 1 h, phosphorylation of ERα Ser167 was determined by western blotting. The phosphorylation levels of ERα Ser167 were ~58 and ~20% higher in cells treated with in 1, and 10 nM rapamycin, respectively, compared with that in vehicle-treated MCF-7 control cells (Fig. 1C). By contrast, following culturing in the presence of 1,000 nM rapamycin for 1 h, the intracellular phosphorylation level of ERα Ser167 in LTED cells decreased to ~50% of that observed in vehicle-treated control cells (Fig. 1C).

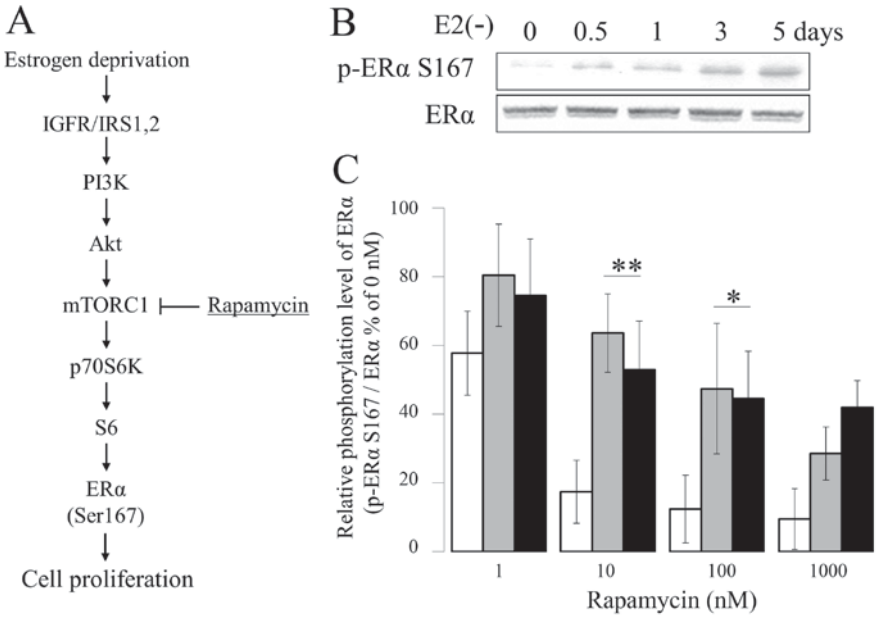

Figure 1. Suppressive effect of Eve on ERα Ser167 phosphorylation. (A) Schematic diagram of activated PI3K/Akt pathway in E_2-deprivation breast cancer. (B) Phosphorylation of ERα Ser167 in MCF-7 cells cultured in E_2-deprived medium for 0-5 days. Calcium dependency of the degradation of microsomal aromatase in the presence of cytosol. MCF-7 (white bar), E_2-deprived (5 days) MCF-7 (gray bar), and LTED (black bar) MCF-7 cells were incubated with the indicated concentration of rapamycin for 1 h (n=4). *P<0.01. Eve, everolimus; ERα, estrogen receptor-α; E_2, estradiol; LTED, long-term E_2-deprived; PI3K, phosphoinositide 3-kinase; Akt, RAC serine/threonine-protein kinase; mTORC1, mechanistic target of rapamycin complex 1; IGFR, insulin-like growth factor receptor; IRS-1, insulin receptor substrate 1.

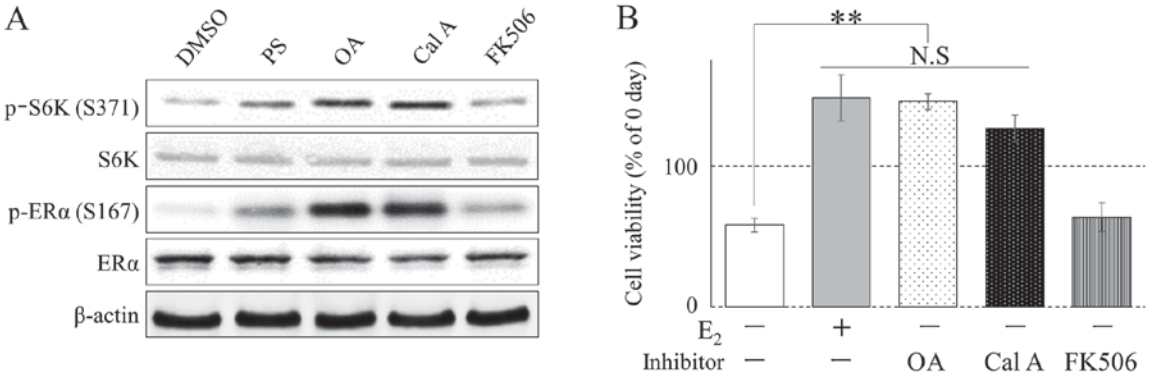

Figure 2. Alteration of the phosphorylation status of ERα and S6K in MCF-7 cells by phosphatase inhibitors. (A) MCF-7 cells were incubated with PS, OA (100 nM), Cal A (1 nM), FK506 (10 nM), or DMSO (0.1%, vehicle) in E_2 (10 nM) medium. Phosphorylation was determined by western blotting. (B) MCF-7 cells were cultured with E_2 (10 nM), OA (100 nM), Cal A (1 nM), FK506 (10 nM), or DMSO (0.1%, vehicle) in phenol red-free RPMI 1640 medium supplemented with 10% dextran-coated charcoal fetal bovine serum for 5 days (n=4). Cell viability was analyzed using Cell Counting kit 8. *P<0.01, **P<0.001. ERα, estrogen receptor-α; DMSO, dimethyl sulfoxide; S6K, S6 kinase; OA, okadaic acid; Cal A, calyculin A.

PP2A inhibition leads to resistance to E_2 depletion via ERα Ser167 phosphorylation. Protein phosphorylation status is determined by the balance between phosphorylation and dephosphorylation. Previous studies have revealed that the mechanism of endocrine resistance involves aberrant signaling through the PI3K/Akt/mTOR signaling pathway (7,12). However, the identity of the phosphatase involved in ERα phosphorylation remains unclear. Western blot analysis was conducted using several protein phosphatase inhibitors, which have been well characterized in phosphorylation studies (17). At 1 h after the addition of each inhibitor [Phos STOP (PS); protein phosphatase inhibitor cocktail, OA and Cal A; PP2A inhibitor, FK506; protein phosphatase type 2B inhibitor], phosphorylation of ERα Ser167 was increased in the culture solution following PS, OA, FK506 and Cal A treatment (Fig. 2A). In addition, OA and Cal A treatment increased the number of cells in the E_2-free medium (Fig. 2B).

E_2 deprivation reduces PP2A levels in MCF-7 cells. PP2A is involved in endocrine therapy resistance (28). Therefore, MCF-7 cells cultured without steroids were examined after 1 or 5 days, which activated mTOR. Levels of phosphorylated ERα Ser167 were analyzed by western blotting. Phosphorylation of ERα Ser167 in LTED cells was induced by long-term E_2 deprivation in MCF-7 parental cells. ERα Ser167 phosphorylation in MCF-7 cells cultured under E_2 depletion for 1 day with LTED was increased 6-fold,

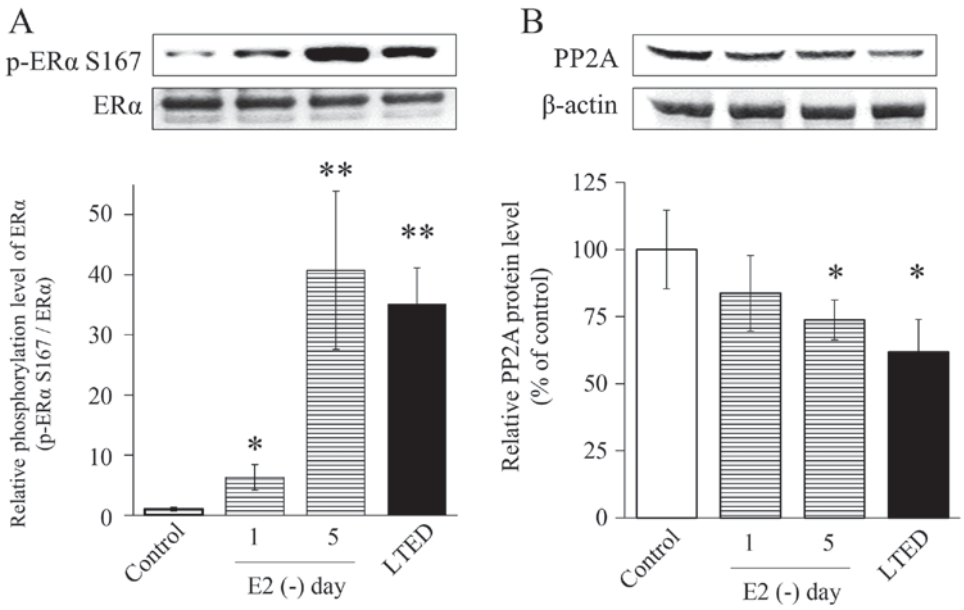

Figure 3. Effect of E_2 deprivation on phosphorylated ERα and PP2A protein levels. (A) MCF-7 cells were cultured without E_2 for 0, 1, or 5 days or under LTED. (B) Phosphorylated ERα Ser167 and PP2A protein levels were analyzed by western blotting; *P<0.01, **P<0.001. ERα, estrogen receptor-α; PP2A, protein phosphatase 2A; E_2, estradiol; LTED, long-term E_2-deprived.

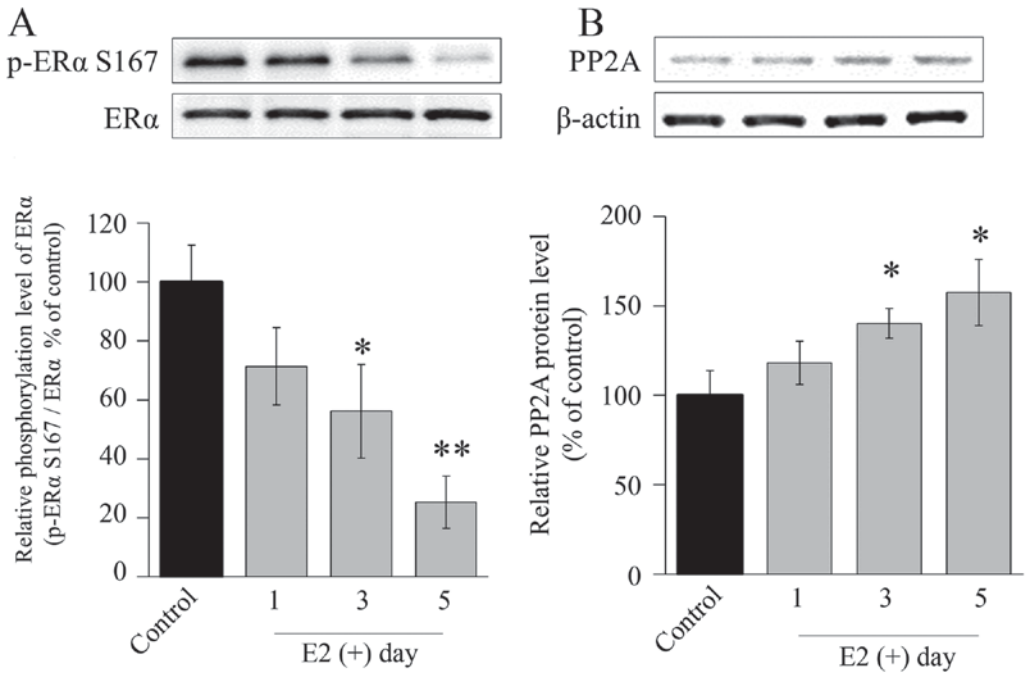

Figure 4. Effect of E_2 on the PP2A protein level. LTED cells were cultured with E_2 for 0, 1, 3 or 5 days. (A) Phosphorylated ERα Ser167 and (B) PP2A protein levels were analyzed by western blotting; *P<0.01, **P<0.001. E_2, estradiol; PP2A, protein phosphatase 2A; LTED, long-term E_2-deprived; ERα, estrogen receptor-α.

whereas that in MCF-7 cells cultured under E_2 depletion for 5 days with LTED increased by 35-fold or more, relative to untreated cells (Fig. 3A). By contrast, after 1 day without E_2 in the medium, PP2A protein levels decreased to 60% of the baseline value (Fig. 3B).

PP2A is upregulated by E_2 under LTED conditions. Phosphorylation of ERα Ser167 in LTED cells was reduced by E_2 exposure, consistent with the results observed in the parental MCF-7 cells. ERα Ser167 phosphorylation in LTED cells was significantly decreased by exposure to E_2 in a time-dependent manner (Fig. 4A). However, 3 days after addition of E_2 to the medium, PP2A protein levels were significantly increased (Fig. 4B).

PP2A activation enhances the effect of Eve. Following E_2 treatment, PP2A expression was increased in the medium. These results indicated that PP2A expression was modulated

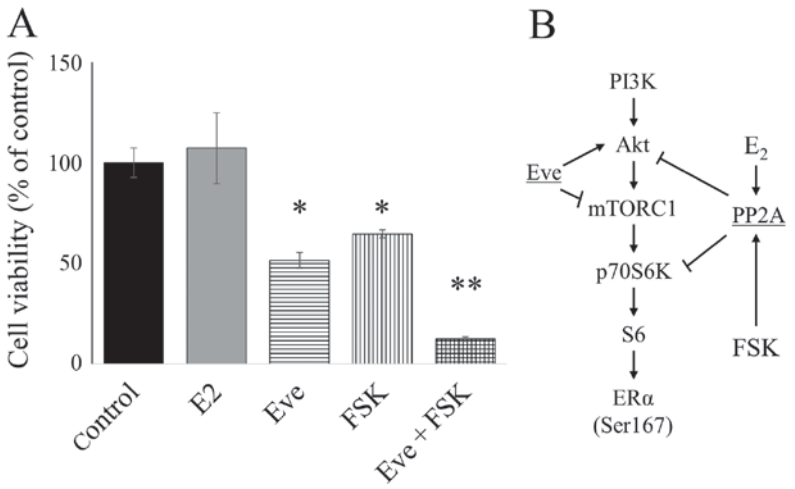

Figure 5. Combination of Eve and FSK reduced the viability of LTED cells. (A) Cell Counting kit-8 assay showing the effect of E_2 (10 nM), Eve (10 nM), and FSK (5 μg/ml), alone or in combination, in LTED cells; *P<0.01, **P<0.001. (B) Schematic diagram of the signaling pathway in E_2-deprived breast cancer cells, showing the effects of Eve, FSK and E_2. Eve, everolimus; FSK, forskolin; E_2, estradiol; LTED, long-term E_2-deprived; PI3K, phosphoinositide 3-kinase; Akt, RAC serine/threonine-protein kinase; mTORC1, mechanistic target of rapamycin complex 1.

by E_2 and has a major role in resistance to E_2 depletion. Therefore, we hypothesized that PP2A activation increases the effect of Eve. FSK is an activator of PP2A. E_2 induced cell growth in ERα-positive LTED cells. To investigate the role of PP2A in this process, the potential role of PP2A activation by FSK in cell death was determined. LTED cells in E_2-depleted medium were treated with Eve (10 nM) and/or the PP2A activator FSK (5 mg/ml) for 5 days. FSK and Eve significantly inhibited cell growth. In addition, the combination of Eve and FSK significantly reduced cell growth (Fig. 5A). E_2 did not suppress the growth of LTED cells despite increased PP2A expression (Fig. 5A).

Discussion

Resistance to AIs is an important clinical problem in oncology. In the present study, PP2A was demonstrated to be an important inhibitory factor for signal activation via phosphorylation of ERα Ser167 in breast cancer MCF-7 cells. PP2A activation by FSK increased the appearance of LTED the effect of Eve. On the basis of these results, it was suggested that FSK may serve an auxiliary role in treating AIs-resistant breast cancer.

In a typical cell, the functions of nearly one-third of proteins are regulated via phosphorylation, and this process controls various biological functions, including cell division, growth, proliferation, and apoptosis (29,30). Depending upon the physiological requirements of the cell, proteins transiently shift from a phosphorylated to a dephosphorylated state, with the balance controlled by protein kinases and phosphatases (30,31). PP2A, a serine/threonine protein phosphatase, has been previously suggested to be a tumor suppressor protein in AIs-resistant ER-positive breast cancer cells (17,28,32). In the present study, PP2A tumor suppressor activity was first observed upon treatment with OA, a selective but not specific inhibitor of PP2A, which potently promoted resistance to E_2 deprivation in MCF-7 cells. It was subsequently demonstrated that the combination of the PP2A activator FSK and Eve significantly decreased LTED cell viability. The only known targets of OA are the catalytic subunits PP1 and PP2A, which are essential components of two basic cellular functions: Growth and cell division (31,33). Previously, co-immunoprecipitation and *in vitro* pull-down assays revealed a direct association between the PP2A-B55 holoenzyme, and Akt; the selectivity of the holoenzyme regulates Akt Thr308 phosphorylation (34).

Our previous study indicated that inhibition of PP2A significantly increases ERα phosphorylation (17). Furthermore, the present study demonstrated that expression of PP2A decreased in response to E_2 depletion. As presented in Fig. 1, the responsiveness of Erα phosphorylation to rapamycin in MCF-7 and LTED cells after 5 days of E_2 depletion was poor. This result indicates that inactivation of S6K was slowed by the reduction of PP2A expression (35). Owing to its substantial effect on ERα phosphorylation, the reduction in PP2A levels is considered to contribute to the abnormal activation of IGF-I receptor/insulin receptor substrate-2 or promote AIs-acquired resistance (36). Cancerous inhibitor of PP2A is a novel oncogene that is frequently overexpressed in breast cancer, and has been reported to be downregulated by the phytoestrogen genistein, which has a high affinity for the estrogen receptor (32).

Eve induces Akt activation. PP2A is an important molecule for Akt suppression, however is downregulated in the E_2 depleted state. The present study suggested that PP2A activation by FSK is a means to eliminate the effect of the decrease in PP2A levels, and it may be effective to use FSK in addition to the AIs and Eve combination (Fig. 5B). The present study supports the previous implication of PP2A (37) as a therapeutic target in AI-resistant breast cancer.

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